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Simultaneous Determination Three Metabolic Regulators of Clomiphene, Trimetazidine, and Meldonium in Food by High Performance Liquid Chromatography Tandem Mass Spectrometry

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ABSTRACT

A liquid chromatography-tandem triple quadrupole mass spectrometry method was developed to determine the residues of clomiphene, trimetazidine and meldonium in food. Samples were extracted ultrasonically with formic acid water-acetonitrile solution (25:75, *V/V*). After MCX Solid Phase Extraction Column Cleanup, the Shisedo PC HILIC column was used for separation. The positive ion multiple reaction monitoring mode was used and the isotope internal standard method was used. The limit of detection (LOD) for clomiphene and trimetazidine was both 0.25 μg/kg. The limit of quantification (LOQ) was 0.5 for both clomiphene and trimetazidine. The LOD for Meldonium was 2.5 μg/kg and the LOQ was 5.0 μg/kg. Recovery rates ranged from 80.1% to 119.9%, and intermediate precision (RSD) was 1.95% to 15.4% (n=6). The method provides a means of convenient and practical quantification of clomiphene, trimetazidine, and meldonium in various food matrices with high accuracy and high reproducibility.

1. INTRODUCTION

Clomiphene, trimetazidine and meldonium are three clinically common drugs, that were listed as prohibited substances by the World Anti-Doping Agency in 2020, and long-term use can cause damage to human health. Clomiphene is an anti-estrogen substance that improves myocardial energy metabolism and helps enhance energy in myocardial cells. It exerts regulatory effects on the body's metabolism by stimulating the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) through the release of gonadotropin-releasing hormone, and regulating the menstrual cycle and inducing masculinization in the organism(Detry et al., 1994; Liepinsh et al., 2006; Pupure et al., 2010; Tuunanen et al., 2008). Trimetazidine can improve cardiac function and enhance the body utilization efficiency of oxygen to generate more energy supply in clinical practice. When used in high doses, The high doses of trimetazidine can enhance athletic performance by improving respiratory function, increasing oxygen supply capacity, inducing mental stimulation, and boosting physical strength in athletes. Meldonium acts on the intracellular mitochondria and shows the ability to improve myocardial energy metabolism at the cellular level. It leads to a reduction in lactate and urea levels in the blood, increases glycogen levels, and subsequently enhances endurance and aerobic exercise capacity. Meldonium also facilitates faster recovery, stimulates the central nervous system, and alleviates stress, thus exerting beneficial effects on overall physical fitness and well-being(Denys & Ihor, 2019; Euler et al., 2022; Wang et al., 2022).The World Anti-Doping Agency (WADA) explicitly listed clomiphene, trimetazidine, and meldonium as prohibited substances in the 2020 List of Prohibited Substances and Methods (S4 category). However, dietary intake of food-derived metabolic regulators may occur in people's daily diet. Some metabolic regulators are naturally present in food, while others may be added during food processing. As potential sources of pharmaceutical exposure, these metabolic regulators can enter the human body and persist within it through the food chain (Eiden et al., 2020). In a study by Euler Luisa, the egg production rate of chickens was significantly enhanced by taking clomiphene oral liquid. However, the residues of clomiphene were found in their eggs(Zahid, Arshad, Zafar, & Al-Mohannadi, 2016). Clomiphene and meldonium were also used in animal feed to improve the reproductive ability and promote body growth.

Therefore, people are likely to have adverse effects on the body after eating these foods by accident.(Minners et al., 2000; Sjakste, Gutcaits, & Kalvinsh, 2005; Temerdashev, Azaryan, & Dmitrieva, 2020; Vilskersts et al., 2009; Wisel et al., 2009; Zakharenko, Petrovsky, & Putilov, 2018). Therefore, the accurate determination of clomiphene, trimetazidine, and meldonium in food is crucial to ensuring fairness and integrity in major sporting events.

However, the existing detection methods for clomiphene, trimetazidine, and meldonium mainly involve urine, plasma, feed, and other matrices, and no specific detection methods for these compounds in food have been established in current international food safety national and local standards.(Pupure et al., 2010; Shaforostova, Gureev, Volodina, & Popov, 2022; Sjakste et al., 2005). Therefore, developing a sensitive, simple, and rapid detection technology for the residues of clomiphene and other metabolic regulators in food matrices is essential. It will not only enhance the level of food quality and safety risk monitoring but also prevent potential health hazards caused by the ingestion of Clomiphene, trimetazidine, and meldonium. The pretreatment process of food is critical before instrumental analysis because of the matrix effect. In this paper, the MCX pass-through solid phase extraction column was used for cleanup. It can integrate extraction, concentration and purification, effectively remove impurities such as fat and phospholipids in the sample matrix, simplifies the pretreatment process of the experiment, and improves the accuracy of analysis(Hmelnickis et al., 2008; Shaforostova et al., 2022). High-performance liquid chromatography with UV detection, liquid chromatography tandem mass spectrometry, and gas chromatography-mass spectrometry is commonly used in food inspection. (Hmelnickis et al., 2008; Peng et al., 2010; Pirok, Stoll, & Schoenmakers, 2019; Shaforostova et al., 2022; Van Thuyne, Van Eenoo, & Delbeke, 2006) Meldonium has the characteristics of simple structure, no ultraviolet absorption, and high polarity, which makes the determination results of liquid chromatography inaccurate and prone to false positives. The detection range of gas chromatography-mass spectrometry is narrow, the operation is complicated, and the detection time. Liquid chromatography-mass spectrometry in tandem technology has the characteristics of high sensitivity, strong anti-interference ability, and a wide range of analysis, which meets the rapid and efficient requirements of current food safety testing.(Gilar, Bouvier, & Compton, 2001; Jiang et al., 2020; Van Thuyne et al., 2006)

This study developed a novel LC-MS/MS method to detect clomiphene, trimetazidine, and meldonium in food using a combined internal standard approach. The extraction process involved the using formic acid water-acetonitrile solution (25:75, V/V) and purification using an MCX solid phase extraction column. Separation was achieved using a Shiseido PC HILIC column (2.0 mm × 150 mm, 5μm). High-performance liquid chromatography-tandem mass spectrometry was utilized for determination. The developed method exhibited excellent purification efficacy, high sensitivity, strong selectivity, and good separation. It can be used for large-scale and precise detection of clomiphene and other three metabolic regulators in food, with the benefits of short detection time and ease of implementation.

2. MATERIALS AND METHODS

2.1 Materials

HPLC-MS/MS equipment used was an Agilent 6470 triple quadrupole tandem mass spectrometer equipped with an Agilent 1290 liquid chromatograph (Agilent, Washington, USA) and PC HILIC column (2.0 mm × 150 mm, 5μm, Shiseido, Tokyo, Japan). MCX solid phase extraction cartridge (3cc/60mg) was purchased from Waters (New York, USA). All samples were purchased from different local supermarkets in Beijing (China) and were stored at 4 °C.

Acetonitrile (Merck, Munich, Germany) and methanol (Aladdin, Shanghai, China) were HPLC grade; All other reagents were of at least analytical grade from Aladdin Chemical Factory (Shanghai, China). Water was obtained using a Milli-Q water purification system (Millipore, USA). The standards of Clomiphene, Clomiphene-D_5, Trimetazidine, Trimetazidine-D_8, Meldonium, Meldonium-D_3 were purchased from Alta Technology Company (Tianjin, China).

The standard stock solutions of Clomiphene, Clomiphene-D_5, Trimetazidine, Trimetazidine-D_8, Meldonium, and Meldonium-D3 were prepared in HPLC-grade methanol at 10 μg mL^{-1} and stored in brown volumetric flasks at -18 °C. The internal standard was prepared at 0.1 μg mL^{-1} (Meldonium-D_3， 2.5 μg mL^{-1}) in HPLC grade methanol in brown volumetric flasks and stored at 4 °C. The mixed working solutions were obtained by diluting the standard stock solutions with HPLC grade methanol to obtain the serial concentrations of 0.5-10 ng mL^{-1} (Meldonium, 5-100 ng mL^{-1}).

2.2 Sample preparation

All samples were purchased from different local supermarkets in Beijing (China) and were stored at 4 °C.

Solid and semi-solid samples. The sample (1.0 g) was accurately weighed into a 10 mL centrifuge tube. Added 20 μL of internal standard solution was added and mixed well, after which it was allowed to stand for 30 min. 5 mL of 0.1% formic acid-acetonitrile solution (25:75, V/V) was then added, and the mixture was shaken vigorously for 30 s before being subjected to ultrasonic extraction for 10 min. The sample was then centrifuged at 10,000 g for 10 min at 4 °C, after which the supernatant was transferred to a new centrifuge tube. The residue was extracted again with 5 mL of extraction solution, and the supernatant was collected for purification.

Liquid sample. The sample (1.0 g) was accurately weighed into a 10 mL centrifuge tube with a cover. Added 20 μL internal standard solution, shaking vigorously for 10 s. 9 mL of 0.2% formic acid-acetonitrile solution (25∶75, *V/V*） was added to dissolve the sample, shaken vigorously for 10 s, ultrasonic extracted for 10 min; The sample was then centrifuged at 10,000 r/min for 10 min at 4 °C. The supernatant was carefully collected and subjected to purification.

Grease and its products. The sample (1.0 g) was weighed and placed into a 10 mL glass graduated tube with a cover (the solid sample was first placed in a water bath at 60 °C for 5 min, with occasional shaking to dissolve the fat). Added 20 μL internal standard solution and mixed well for 10 s, followed by adding 5 mL of 0.1% formic acid-acetonitrile solution (25:75, *V/V*) to dissolve the sample, shaking vigorously for 2 min. After the supernatant was stratified, the supernatant was transferred to the second centrifuge tube, and5 ml of extract was added to the residue for repeated extraction. After mixing the supernatant, the supernatant was centrifuged for 10 minutes at 10000 r/min at 4℃, and the supernatant was carefully collected for purification.

Purification. Before extraction, the MCX column was activated by 3 mL methanol and 3 mL water, successively. Then, all of the sample solution was added to the activated MCX column and pushed through the column by air from a pressurized syringe. The flow rate of the sample solution was controlled by about 2-3 mL min^{-1}. After that, the column was rinsed using 2 mL of 0.1% formic acid-acetonitrile solution (25:75, *V/V*) and the target compound was desorbed with

5 mL of a methanol solution containing 2% ammonia water. The eluent was collected, dried under weak nitrogen flow at 37°C , dissolved in 1 mL 70% methanol solution, filtered by 0.22 μm filter, and analyzed by HPLC-MS/MS.

2.3 Methods

The column temperature was 25 °C. The flow rate was 0.3 ml min^{-1} with an injection volume of 2 μl. A 20 mM ammonium acetate aqueous solution (containing 0.05% formic acid) and acetonitrile (70:30, V/V) were used as the mobile phases. Mass spectrometry conditions: electrospray ion source (ESI); positive ion mode scan; drying gas temperature was 350 °C with 5 L min^{-1}; drying gas was N_2; atomizing gas pressure was 275.8 kPa; multi-reaction detection method. The mass spectrometric parameters of the compounds are shown in Table 1.

Table 1. MRM parameters and internal standards of 3 metabolic regulators

Analyte	Precursor ion（m/z）	Production（m/z）	Fragmentation voltage（V）	Collision energy（eV）
Clomiphene	406.1	100*；58.1	160	30；30
Clomiphene -D_5	411	100	160	30
Trimetazidine	267.2	181.1*；166.1	100	15；35
Trimetazidine -D_8	275.1	181.1	100	15
Meldonium	147.1	58.1*；59.2	60	40；20
Meldonium-D_3	150.1	61.2	60	40

*Quantitative ion

3. RESULTS AND DISCUSSION

3.1 Optimization of sample pretreatment

As animal-derived foods are known to contain high amounts of fat and animal protein, methanol as the extraction reagent can result in the simultaneous extraction of fat and protein, thereby affecting the extraction efficiency of the target compound and the subsequent purification

process.(Dong et al., 2015; Wu, Liu, & Peng, 2017). Hence, acetonitrile was chosen as the primary extraction reagent to minimize interference from these components. Because the polarities of clomiphene, trimetazidine, and meldonium are quite different, the extraction effects of 50% acetonitrile aqueous solution, 75% acetonitrile aqueous solution, and acetonitrile on clomiphene, trimetazidine, and meldonium were compared at the same time. The results showed that a 75% acetonitrile solution can extract clomiphene, trimetazidine, and meldonium, and the extraction effect is better without a significant difference, as shown in Figure 1. Requirements: Choose 0.1% formic acid water-acetonitrile solution (25:75, *V/V*) as the extraction reagent for solid samples such as poultry meat and seasoning powder. 0.2% formic acid in acetonitrile solution was used as an extraction reagent for liquid samples such as eggs and milk. Considering that the solid and semi-solid foods may be inhomogeneous, this method examined the recovery effect of beef, fish, sauce, oil, and other types of food after one extraction and two extractions. It was found that the recovery of the extract once is lower, and the recovery is about 75% to 88%. After the extractions twice were combined, the total recovery rate can reach about 89%to 105%, as shown in Figure 2. Therefore, it is determined that solid and semi-solid food should be extracted twice during the extraction process. The sample is uniform for liquid foods with high water content, such as milk and liquid, and the recovery rate of one extraction with the extract can be as high as 90.7% to 97.3%. To reduce the experimental steps, the extraction frequency of liquid high water content food is one time.

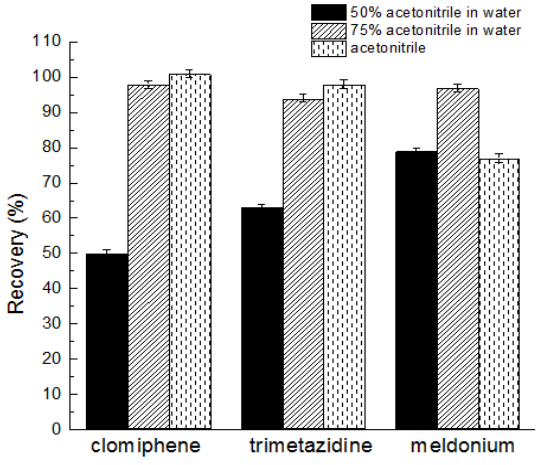

Figure 1. Effects of different extract solvents on the recoveries of clomiphene, trimetazidine, meldonium.

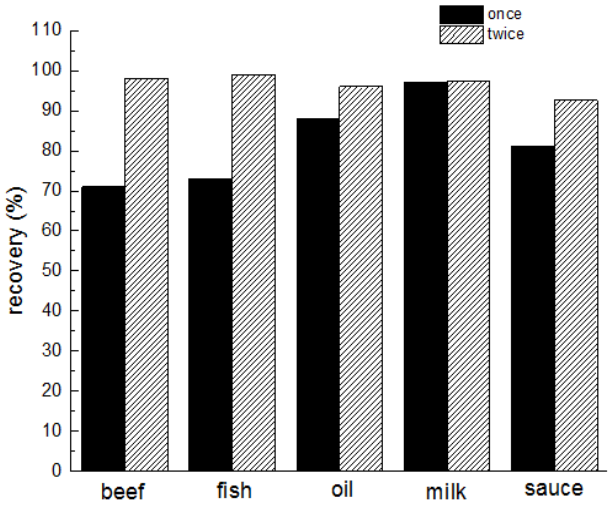

Figure 2. Effects of the number of extraction steps on the recoveries of clomiphene, trimetazidine, meldonium.

3.2 Optimization of purification methods

The purification effects of PRiME HLB (6 cc/200 mg), MCX (3 cc/60 mg) and WAX (3 cc/60 mg) solid-phase extraction column were compared. The results showed that both MCX (3 cc/60 mg) and PRiME HLB (6 cc/200 mg) exhibited average recoveries exceeding 90%, with no significant difference observed between the two columns（P>0.05）. In contrast, WAX (3 cc/60 mg) was unsuitable for the purification of the three metabolic regulators as shown in Figure 3. Additionally, it showed that PRiME HLB had limited effectiveness in purifying certain types of samples, such as seasoning powder, soy sauce, and sugar. In contrast, the MCX column displays dual retention capabilities for both reversed-phase and cation exchange, leading to a strong retention effect for clomiphene, trimetazidine, and meldonium (Figure 4). Thus, the MCX (3 cc/60 mg) solid-phase extraction column was selected as the pre-treatment purification method for basic compounds in complex food matrices. Based on the packing characteristics of MCX, a methanol solution containing 2% ammonia was chosen as the elution solvent for this type of solid-phase extraction column.

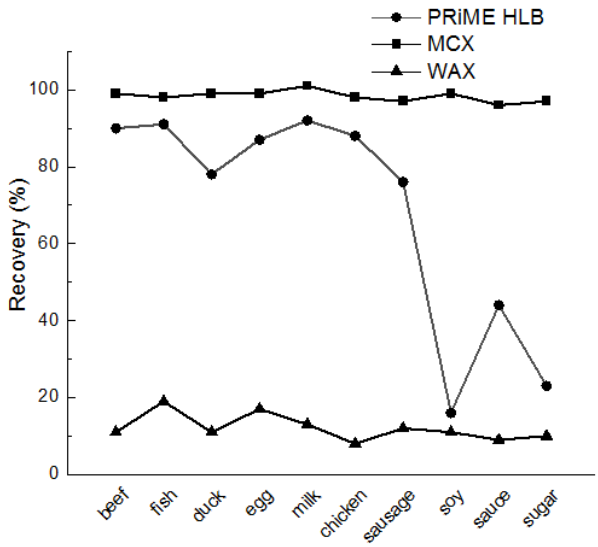

Figure 3. Comparison of the purification efficiency of different materials.

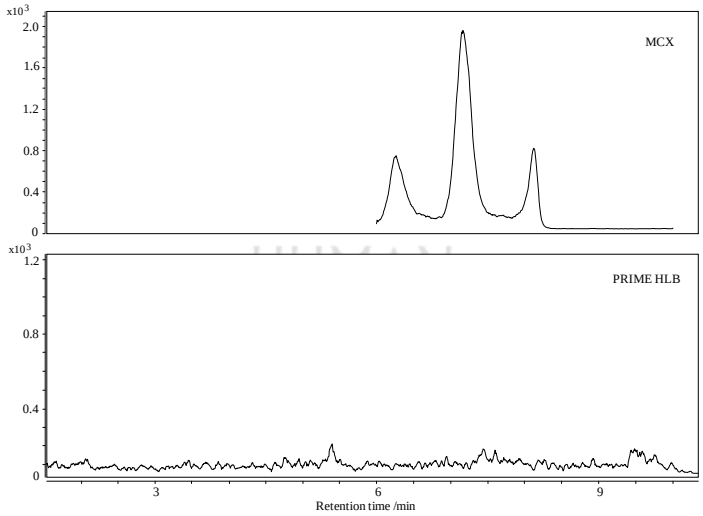

Figure 4. Comparison chart of purification effect of meldonium with MCX and PRiME HLB in soy sauce sample.

3.3 Optimization of mass spectrometry conditions

Based on the chemical properties of clomiphene, trimetazidine, and meldonium, and their internal standards, the ESI^+ ionization mode was selected for the mass spectrometry analysis. The mass spectrometer conditions were optimized using a flow syringe pump for continuous injection. The experimental results revealed that the ESI^+ ionization mode provided a higher abundance of precursor ions for the target compounds in the ion source, indicating its suitability

for analyzing these compounds. The secondary mass spectrometry analysis was conducted using production scanning, and product ions were selected for determining quantitative and auxiliary qualitative ions. Mass spectrometry parameters, such as fragment, cell accelerator voltage, collision energy, and Dell, were optimized to maximize the ion pair intensity generated by the quasi-molecular ion and characteristic fragment ion of the target compound. Following the regulations outlined in EU Resolution No. 2002/657/EC, two pairs of characteristic transitions with higher response values were chosen for each target as quantitative and qualitative transitions. various mass spectrometry parameters were optimized in the multiple reaction monitoring modes. The relevant parameters have been summarized in Table 1.

3.4 Optimization of chromatographic conditions

Selection of Column. In this experiment, the Shiseido PC HILIC column (150 mm × 5 mm, 2.0 μm), Hypersil GOLD C_{18} column (50 mm × 4.6 mm, 1.9 μm) and Agilent Poroshell 120 PFP column (100 mm × 4.6 mm, 2.7 μm) were performed to separate the target compounds. As a result of the above three columns, three target compounds were effectively separated. As shown in Figure 5, PC HILIC columns were selected as the analytical columns for this method to fully separate meldonium from complex matrices due to the strong polarity of meldonium.

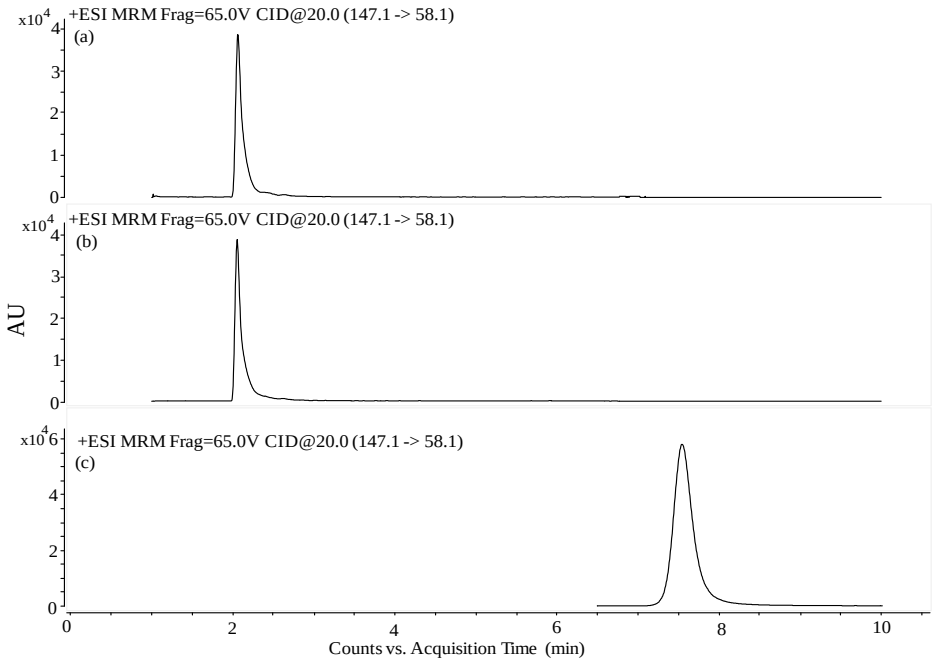

Figure 5. Retention Effects of Different Chromatographic Columns on Meldonium

Agilent Poroshell 120 PFP(a), Hypersil GOLD C_{18}(b), and Shiseido PC HILIC(c).

Selection of mobile phase system. The selection of a mobile phase can significantly impact the ionization of the target compound and the sensitivity of mass spectrometry detection. In PC HILIC columns, mobile phases typically consist of acetonitrile and aqueous ammonium acetate. The pH values of the mobile phase can also affect the separation and retention of target compounds, as well as the results of mass spectrometry ionization. The separation effects of different mobile phases on the target compounds were compared, including 20 mM ammonium acetate aqueous solution - acetonitrile (30:70, *V/V*) which contained 0%, 0.025%, 0.05% formic acid. It was observed that the 0% formic acid mobile phase resulted in peak shape broadening and inadequate elution of clomiphene. The separation of the three target compounds was similar between the 0.025% formic acid and 0.05% formic acid mobile phases. However, the mobile phase exhibited a higher separation effect. Therefore, in this study, the three metabolic regulators were separated on the PC HILIC column using 20 mM ammonium acetate aqueous solution (containing 0.05% formic acid) - acetonitrile (30:70, V/V) as mobile phase. The effects of different chromatographic columns and mobile phases on the separation of the three metabolic regulators are shown in Figures 6-8.

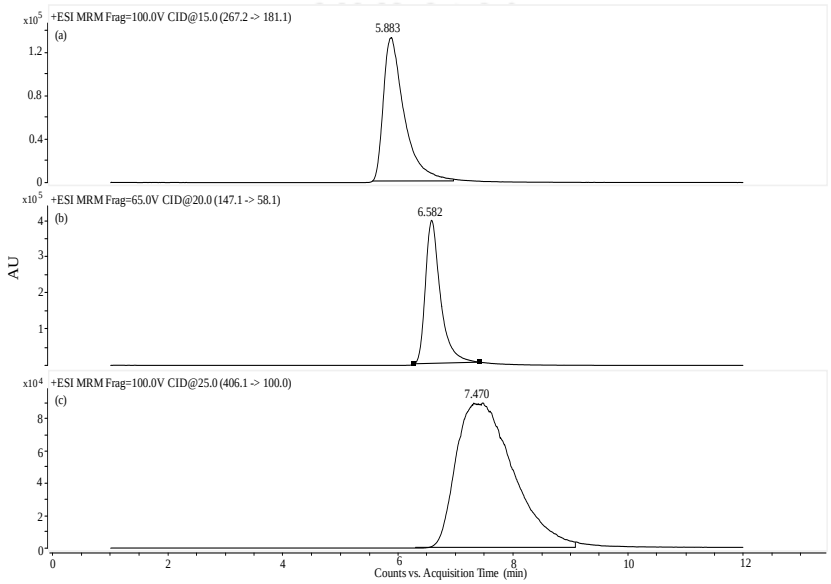

Figure 6. Standard solution MRM chromatograms of three metabolic conditioners in mobile phase 1. (a) trimetazidine, (b) mildronate, (c) clomiphene.

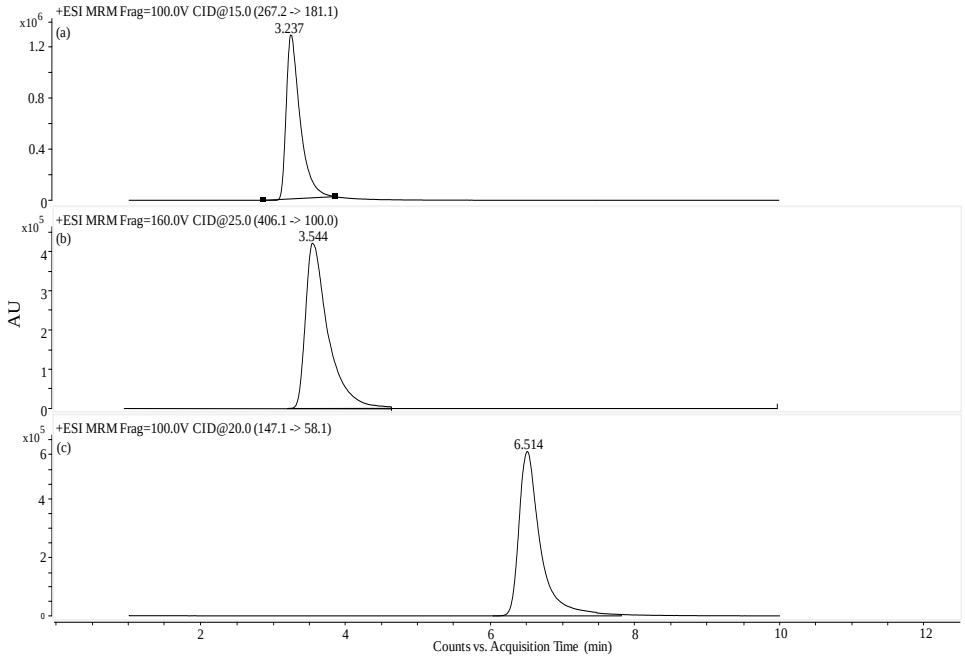

Figure 7. Standard solution MRM chromatograms of three metabolic conditioners in mobile phase 2. (a) trimetazidine, (b) clomiphene, (c) mildronate.

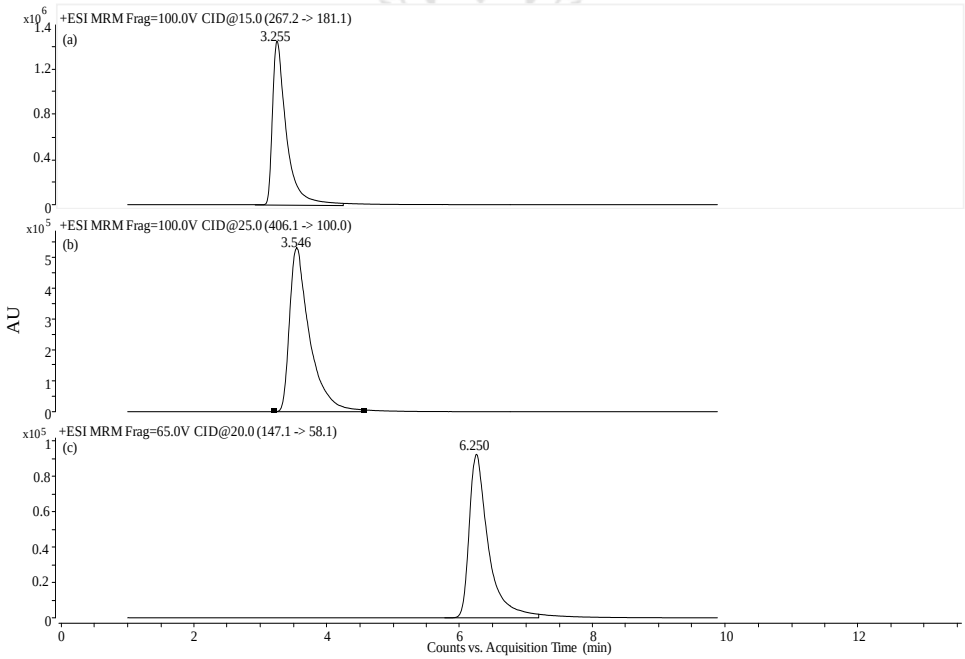

Figure 8. Standard solution MRM chromatograms of three metabolic conditioners in mobile phase 3.(a) trimetazidine, (b) clomiphene, (c) meldonium.

Selection of quantitative methods. In this study, the matrix effect (ME), which refers to the influence of other components in the product other than the target analyte on the measured value of the analyte was evaluated. Even though the sample was purified to a certain extent, the matrix effect could not be eliminated. As shown in Table 2, it was observed that meldonium exhibited a strong matrix inhibition effect after sample treatment. To mitigate the matrix effect, an isotope internal standard was added, which is a common method used in eliminating matrix effects. Furthermore, an isotope internal standard was chosen to accurately quantify the target compounds due to the complexity of the food matrix. After using the internal standard, the ME of the three metabolic regulators ranged from 0.85 to 1.15. Therefore, it was evident that the internal standard method can improve the stability of the detection method, as depicted in Table 3.

Table 2. Matrix effects of 3 metabolic regulators (without internal standard)

Analyte	trimetazidine	clomiphene	meldonium
milk	0.81	0.75	0.73
beef	0.89	0.82	0.11
sauce	0.92	0.67	0.29
fish	0.83	0.90	0.30
oil	0.87	0.77	0.54

Table 3. Matrix effects of 3 metabolic regulators (with internal standard)

Analyte	trimetazidine	clomiphene	meldonium
milk	0.89	0.95	0.93
beef	0.91	0.89	0.99
sauce	0.90	0.99	0.89
fish	0.93	1.01	1.02
oil	0.98	0.95	0.98

3.5 Linearity range and detection limit

The contents of clomiphene and trimetazidine were 0.500, 1.00, 2.00, 5.00, and 10.0 μg-1, respectively, prepared with 70% methanol aqueous solution with mixed standard working solution and internal standard working solution. Mixed standard series solutions with meldonium content of 5.0, 10.0, 20.0, 50.0, and 100.0 μg L^{-1} respectively. The internal standard concentrations of clomiphene, trimetazidine, and meldonium were 2 μg L^{-1}, 2 μg $L^{-1,}$ and 50 μg

L^{-1}, respectively. A standard curve was constructed using the peak area ratio (y) of the target compound and the corresponding internal standard against the concentration ratio (x). The linear range exhibited excellent linearity with a correlation coefficient (r^2) greater than 0.9991 for all three metabolic regulators. To determine the lower limit of quantification, a blank sample was selected, and the standard solution was added quantitatively. The processed sample was measured using the experimental method, and the minimum detectable amount was determined as the concentration where the qualitative ion signal-to-noise ratio of the spectrogram was greater than 3. The lower limit of quantification for each compound was calculated, and the results are presented in Table 4.

Table 4. Linearity, LOQ, and LOD of three metabolic modulators

Analyte	Linear equation	Linear range (μg kg^{-1})	r^2	LOQ (μg kg^{-1})	LOD (μg kg^{-1})
Clomiphene	Y=9.45X-0.3315	0.5-10.0	0.9998	0.5	0.2
Trimetazidine	Y=12.28X+0.6012	0.5-10.0	0.9993	0.5	0.2
Meldonium	Y=0.90X-0.0068	5.0-100	0.9991	5.0	2.0

3.6 Recovery rate and precision of the method

To assess the accuracy and precision of the method, the recovery expressed as a percentage and the relative standard deviation (RSD) were calculated using the peak area ratios of the targets to the internal standard. Five matrices, including beef, fish, oil, milk, and sauce, were selected for the standard addition experiment. To better investigate the recovery rate and precision of the method, the experiment was performed on blank matrices at 1-fold, 2-fold, and 10-fold recovery. Each concentration level was subjected to six parallel experiments. As shown in Table 5, the average recovery at different concentrations ranged from 80.1% to 119.9%, with RSD of 1.95% to 15.4%.

Table 5. Recoveries and RSD of the three metabolic regulators (n=6)

		beef	fish	milk	oil	sauce
Analyte	Concentration （μg kg^{-1}）	recovery (%) / RSD	recovery (%) / RSD	recovery (%) / RSD	recovery (%) / RSD	recovery (%) / RSD
clomiphene	0.5	99.9(1.5)	99.9(1.6)	99.8(4.45)	99.9(2.3)	100.2(2.7)
	1	92.1(1.8)	92.1(1.8)	101.9(11.3)	101.9(9.5)	105.5(8.8)
	5	99.0(2.3)	99.0(2.3)	99.7(3.4)	100.0(2.2)	98.9(3.0)
trimetazidine	0.5	98.9(2.0)	100.5(1.5)	100.8(2.1)	101.5(3.4)	100.6(1.5)
	1	104.9(9.8)	96.9(6.3)	105.3(2.3)	99.8(2.1)	106.9(7.0)
	5	99.8(2.7)	100.0(1.7)	98.4(1.1)	95.3(3.9)	100.0(3.6)
meldonium	0.5	101.5(3.6)	101.2(3.4)	99.2(2.0)	101.5(4.2)	98.7(2.3)
	1	99.1(5.4)	101.4(5.9)	98.3(7.6)	100.9(5.8)	97.6(4.4)
	5	100.2(4.4)	99.2(4.5)	99.7(2.4)	97.7(3.1)	99.8(3.4)

4. CONCLUSION

In this study, a liquid chromatography-tandem mass spectrometry method was developed for the simultaneous detection of clomiphene, trimetazidine, and meldonium in food. The acetonitrile-formic acid aqueous solution was used as an extraction reagent, and effective extraction of clomiphene, trimetazidine, and meldonium from the sample was achieved. The MCX (3 cc/60 mg) solid phase extraction column was employed to purify the sample and eliminate impurities. The use of the isotope internal standard method effectively reduced the matrix effect and ensured the accuracy of the results. The recovery of the method at different concentration addition levels was 80.1 to 119.9%, and precision RSD was from 1.95 to 15.4% (n=6). The method was simple and easy to perform, and provided fast and efficient analysis, enabling accurate quantification of clomiphene, trimetazidine, and meldonium in food. This method exhibited important application value as a detection basis and rapid screening method for monitoring drug residues of clomiphene, trimetazidine, and meldonium in food. Furthermore, this study provides a foundation for further research on the abuse of metabolic regulators in food.

Acknowledgments

None.

Author contributions

Hao Wang: methodology, investigation, formal analysis, and writing the original draft; Yu Wang: formal analysis, methodology, and writing review& editing; Ge Ge: writing review & editing; Li Lin: methodology and writing – review & editing; Tongna Mu: supervision and writing review & editing; Wenchao Zhang: supervision and writing review & editing.

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